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Molecular and genetic analysis in the model organism *Caenorhabditis elegans* was used to identify new negative regulators of tyrosine kinase/ras mediated signal transduction pathways that are candidate tumor suppressors. A nematode homolog of the cbl proto-oncogene was shown to act by regulating activation of Ras by an epidermal growth factor receptor homolog (LET-23). The *rok-1* (*r*egulator *o*f *k*inase-mediated signaling) locus of *C. elegans* was molecularly cloned by correlating genetic and physical maps and rescue of mutant phenotypes in transgenic nematodes. A new locus, *rok-2* was identified in a genetic screen for mutations that hyperactivate the LET-23 signaling pathway in the absence of SLI-1 function. Genetic interactions among these nematode negative regulatory mutations were examined by the construction of multiple mutant strains; these results suggest that multiple pathways regulate the tyrosine kinase/ras signal transduction. These results will help elucidate the function of the cbl family of proto-oncogenes, and to identify novel candidate tumor suppressor loci.

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Introduction

An important short-term goal of breast cancer research is to identify candidate genes for identifying precancerous cells and cancerous cells. Ideally, precancerous or cancerous cells could be characterized by a set of molecular markers. These markers would best be chosen from the set of genes altered during early stages of precancer formation. It is also crucial to understand the cellular regulatory pathways in which these genes act. *C. elegans* molecular genetics provides a facile model system with which to identify and to examine gene interactions *in vivo* [reviewed by Sternberg, 1993]. For example, it has been clearly shown that the *C. elegans* ras protein LET-60 acts downstream of EGF-receptor homolog LET-23 [Han & Sternberg, 1990; Aroian et al. 1990], that the adaptor protein SEM-5 acts between LET-23 and ras [Clark et al., 1992a; Katz, et al., submitted] and that the LIN-45 raf protein acts downstream of LET-60 ras [Han et al., 1993]. This universal signaling pathway is the target of many mutations contributing to oncogenesis in humans. Overexpression or activation by mutation of LIN-3, LET-23, LET-60 results in excessive vulval differentiation; thus activation of the homologous genes leads to cancer in humans and vulval differentiation in nematodes. Therefore, negative regulators of vulval differentiation defined by loss-of-function mutations that lead to excessive signaling are analogous to tumor suppressor loci.

Using the powerful genetics of *C. elegans*, we have identified five pathways of negative regulation of LET-23 mediated signaling have been identified. We are carrying out genetic screens that can identify additional genes involved in negative regulation, either in known pathways or in new ones. Our genetic studies have identified several negative regulators of LET-23-mediated signaling in *C. elegans* [reviewed by Sternberg, 1994]: SLI-1 [Jongeward et al., 1995; Yoon et al., 1995], UNC-101 [Lee et al., 1994], ROK-1 [see details below], LIN-15 [Ferguson & Horvitz, 1989; Huang et al., 1994]. ROK-2 was identified by our screens this past year. In general, these negative regulators are redundant, such that elimination of any one has no effect on the normal signal transduction. In the absence of two regulators, excessive vulval differentiation occurs. These genetic properties are similar to the synergistic action of oncogenic mutations, where several mutations are necessary for a phenotypic change. However, such synthetic mutations are difficult to study except in genetically facile organisms such as *C. elegans*.

If we can identify many loci possibly involved in the analogous processes in *C. elegans*, and identify human homologs, this will help human geneticists in several ways. First, this will provide candidate genes for the positional cloning of tumor suppressor loci defined by human cancer genetics. Second, this will provide molecular probes with which to examine tumorous tissue for alterations. The sooner we can identify the many potential tumor suppressor loci, the more effectively that analysis of the role of tumor suppressor mutation in breat cancer initiation and progression can be assessed. Current technology easily allows parallel processing of samples, and is thus limited by the number of molecular probes. Lastly, we can link together tumor suppressor genes in functional pathways, much as we have been able to do for the LET-23-mediated pathway. If there are multiple pathways of tumor suppressor gene action, then we need to know how to intervene in each one.

One implication of multistep carcinogenesis is that synergism occurs between mutations. The genetics of the negative regulators that we have identified is analogous: mutation of SLI-1 or ROK-1 alone causes no defect, yet inactivation of both leads to increased signaling. The roles of such apparently redundant genes are difficult to study except in powerful genetic systems; *C. elegans* vulval differentiation provides such a system.

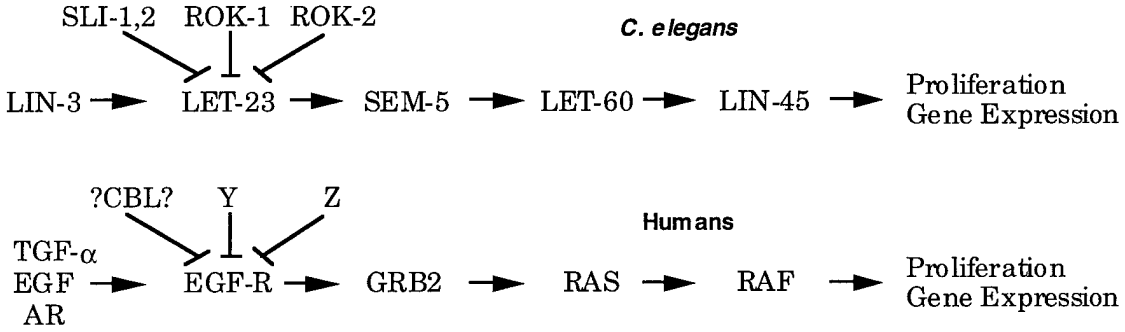


Figure 1. Pathway for the major EGF-R signaling pathway in humans and the analogous LET-23-mediated signaling pathway in *C. elegans*. Arrows indicated positive reguation; bars represent negative regulation. Other components are known.

The oncoprotein cbl has come under intense study during the past year. Notably, a second cbl gene, cbl-b has been identified from huma breast epitheilial cells [Keane et al., 1995]. In addition, cbl has been shown to be phosphorylated and associate with a variety of signal transducing proteins in a number of cell types. This has raised the interest in the basic molecular biology of cbl, and hence of SLI-1. Gratifyingly, the nematode genetic studies that were the basis of this project suggested a direct interaction with EGF-receptor. Indeed several laboratories have demonstrated such an association [D. Bowtell & W. Langdon, submitted; J. Schlessinger, pers. comm.; Hartley et al., 1995; Tanaka et al., 1995].

Progress

The goal of this project is to identify potential tumor suppressor genes and link them into functional pathways with each other, and with known proto-oncogenes and tumor suppressor genes. *C. elegans* vulval differentiation provides a facile model system with which to study EGF-receptor/*c-neu*-mediated signal transduction and its regulation. The *C. elegans sli-1, sli-2* and *rok-1* genes negatively regulate LET-23. We are using *C. elegans* molecular genetics to study and clone these genes in *C. elegans*, and will use molecular biology to clone their human homologs. The specific goals of the project are as follows.

1. **Analyze SLI-1 function** in *C. elegans* through molecular genetics.
2. **Molecularly clone *sli-2*.**
3. **Molecularly clone *rok-1*.**
4. **Identify and clone additional genes acting in concert with *sli-1*, *sli-2*, and *rok-1***
5. **Examine the functional interactions of *sli-1*, *sli-2*, *rok-1* in regulating other conserved signaling pathways.**
6. **Clone human *sli-2*, *rok-1*, and newly identifed genes from human breast tissue libraries** to generate reagents with which to test the hypothesis that these are novel tumor suppressor loci.
7. **Test the functional homology of *c-cbl* and *sli-1*** by introducing the human cDNA into transgenic nematodes defective in *sli-1*.

In the following, progress on goals 1-5 is described.

1. Molecular genetics of *sli-1*

We identified the *sli-1* locus as a negative regulator of LET-23, the *C. elegans* homolog of EGF-R/c-neu/HER3/HER4, using extragenic suppressor analysis [Jongeward et al., 1995]. We cloned the *sli-1* locus by correlating genetic and physical maps and rescuing a *sli-1* mutant in transgenic nematodes [Yoon et al., 1995]. A 10.5 kilobase genomic fragment has the ability to provide all known functions of *sli-1* in transgenic animals, i.e., it rescues the suppression phenotype of *let-23*. In addition, increased copy number of the *sli-1* locus enhances several phenotypes of a *let-23* mutation, supporting our hypothesis that this normally functions as a negative regulator of LET-23-mediated signaling.

c-cbl and *sli-1* both contain a so-called Ring Finger [$CX_2C_{9\text{-}27}CX_{1\text{-}3}HX_2CX_2CX_{6\text{-}48}CX_2C$] putative metal binding finger [Lovering et al., 1993]. The truncated, oncogenic form of *v-cbl* is missing the finger, as is the truncated form found in HUT78 T cell lymphoma cells [Blake & Langdon, 1992]. To determine whether deletion of the ring finger decreases or increases SLI-1 activity, we constructed by site-directed mutagenesis of the genomic clone truncated forms of the SLI-1 protein. We analyzed two constructs in transgenic animals that are defective in endogenous *sli-1*, scoring the phenotypes of *sli-1* mutants: suppression of *let-23* lethal, vulvaless, spicule and P12 defects. We found that there is no dominant effect.

We have isolated more than 12 alleles of *sli-1* [Jongeward et al., 1995]. We will determine the molecular basis of these mutations using techniques standard in my laboratory [Han & Sternberg, 1991; Aroian et al., 1993, 1994; Lee et al., 1994].

These mutations will reveal functionally important regions of SLI-1. Certain *sli-1* alleles display intragenic complementation. For example, *sy102* in *trans* to *sy112* is wild-type for its suppression phenotype. These might be in distinct exons, or in functional domains. We have sequenced the *sy102* and *sy112* alleles, have found several candiate mutations, and are now resequencing to confirm that they are not PCR artefacts.

In collaboration with Dr. Andy Golden [NCI, Frederick], we are expressing *sli-1* in insect cells to make antisera.

To test the hypothesis that *sli-1* mutations bypass the requirement for Ras, strains mutant in a series of *let-60 ras* alleles [Beitel et al., 1990] and *sli-1(sy143)* were constructed. The lethality but not the vulvalessness of *let-60* mutations were suppressed in the F1 generation (Table 1). F2 homozygoetes from homozygous let-60 mothers all died, consistent without previous observations that *sli-1(sy143)* does not suppress complte loss-of-function *let-60* mutations for viability [Jongeward et al., 1995]. This observation indicates that during vulval induction, *sli-1* is regulating ras activation. The interactions of *sli-1* with *sem-5* [Clark et al., 1992a] are now being tested.

Table 1. Suppression of the inviability but not the vulvalessness of *let-60 ras* mutations by a *sli-1* mutation. The phenotypes of F1 homozygous *let-60* animals from heterozygous mothers were determined for four partially defective, recessive *let-60 ras* mutations (Beitel et al., 1990). We anlayzed less informative alleles previously [Jongeward et al., 1995]. *let-60* homozygotes were identifed with a closely linked marker *unc-24*. For viability counts, the total progeny of 10 hermaphrodites of each genotype were counted (approx. 2000 hatched larvae per strain), and the number of Unc (*let-60/let-60*) adults compared to total progeny.

Ras genotype	Viability		Vulval differentiation	
	sli-1(+)	*sli-1(-)*	*sli-1(+)*	*sli-1(-)*
+	100%	100%	3.0	3.0
let-60(n2022)	16	85	2.99	2.95
let-60(n1876)	14	100	0.0	0.052
let-60(n2034)	17	87	0.045	0.086
let-60(n2035)	22	89	0.016	0.034

sli-1 act only in certain tissues [Jongeward et al., 1995]. To determine if this is simply due to it not eing expressed in all tissues, we will examine expression of the *sli-1* gene. We constructed fusion genes with *sli-1* genomic sequences and either *lacZ* or Green Fluorescent Protein (GFP) as reporters [Fire et al., 1990; Chalfie et al., 1994]. Transgenic animals will be analyzed for expression of *sli-1*.

2. Genetics and molecular cloning of *sli-2*

One gene that is likely to act in concert with *sli-1* is *sli-2*, defined by a single allele, *sy262*. *sy262* was isolated as an "unlinked non-complementer" of *sli-1*.

We mapped *sli-2* to *C. elegans* linkage group V, between *unc-46* and *dpy-11*, a region of 2 mu. Of 66 recombinants between *unc-46* and *dpy-11*, 3 recombinants were between *unc-46* and *sli-2*, and 63 were between *sli-2* and *dpy-11*. Therefore, *sli-2* maps closer to *unc-46*. Using 95% confidence intervals estimates, *sli-2* lies in a region spanned by approximately 150 kb of genomic clones. Yeast artificial chromsome (YAC) and cosmid clnoes covering this region have been prepraed. We will now inject cosmid and YAC clones into *let-23(sy1); sli-2(sy262)* animals to test for rescue of the hyperinduced phenotype caused by *sli-2* in this background.

3. Genetics and molecular cloning of *rok-1*

Overview: We identified the *rok-1* (*r*egulator *o*f *k*inase) locus (*IV*) of the nematode *C. elegans* in a genetic screen for new negative regulators of the vulval induction pathway. At 20°C, *rok-1(sy247)* causes essentially no phenotypes in an otherwise wild-type background. However, in combination with mutations in other negative regulator genes, *unc-101*, and *sli-1*, it causes pleiotropic effects including excessive vulval differentiation larval lethality and abnormal spicules. Animals defective in all three negative regulatory genes have a greater extent of vulval differentiation than any single or double mutants. *rok-1(sy247)* causes a slight temperature-sensitive gonad-dependent excessive vulval differentiation. We propose that *rok- 1* defines a fifth negative regulatory activity acting on the vulval induction pathway. *rok-1* differs in other negative regulators in that it appears to be exclusively on stimulated activity of the signaling pathway. The apparent redundancy differs with respect to different phenotypes. We have molecularly cloned the *rok-1* locus by correlating the genetic and physical maps in the *rok-1* region of chromsome IV, and identifying an 11.5 kb genomic clone that complements a *rok-1* mutation in transgenic nematodes.

We suspected for two reasons that there should be more negative regulators in addition to the genes previously identified such as *unc-101* and *sli-1*: 1) molecular analyses of *unc-101* and *sli-1* suggest that the interaction of these two genes may not be direct and that more genes may be involved in this interaction, and 2) the phenotype of *unc-101; sli-1* double mutants is not 100% penetrant, that is, only ~50% of the population of the animals of the genotype *unc-101; sli-1* show excessive vulval differentiation whereas the remaining individuals are wild type, suggesting that there may be additional negative regulators. In this study, we took advantage of the fact that *sli-1* mutations can cause a synthetic Muv phenotype in combination with *unc-101* mutations (Jongeward & Sternberg, 1995). We performed genetic screens where we isolated mutants with excessive vulval differentiation after mutagenizing *sli-1* mutant animals.

Strains and general methods.

Methods for culturing and handling the nematode and general genetic methods were described by Brenner (Brenner, 1974). All genetic experiments were performed at 20 °C except when specified. The standard strain N2 was from Brenner (1974). The markers for linkage mapping and three factor crosses of *rok-1(sy247)* are as follows: LG I: *dpy-5(e61);* LG II: *dpy-10(e128);* LG III: *dpy-17(e164);* LG IV: *dpy-20(e1282), unc-22(s7), unc-31(e169), unc-24(e138), dpy-13(e184), dpy-4(e1166);* LG V: *dpy-11(e224);* LG X: *dpy-3(e27), unc-1(e719), sli-1(sy143).* The alleles for ex-

amining genetic interactions of *rok-1(sy247)* are as follows: *let-23(sy1)* (Aroian and Sternberg, 1991) *unc-101(sy108), sli-1(sy143), lin-15(sy197)* and *lin-15(n744).*

We observed the extent of vulval differentiation using Nomarski optics as described in (Han and Sternberg, 1990). We observed the vulva differentiation of the animals in their L3 molt stage, when the induced VPCs should have divided twice to generate four daughter cells and uninduced VPCs have divided once to generate two daughter cells. The average number of induced VPCs were calculated as the total number of induced VPCs divided by the number of animals observed. The average number of induced VPCs is in wild-type animals is three, and that in Hin animals is greater than three.

To ablate the anchor cell, we performed microsurgery using a laser microbeam. The procedure for microsurgery was described in (Avery and Horvitz, 1987, Sulston and White, 1980). We ablated the Z1, Z2, Z3, and Z4 cells, which include the precursors of the germ line, the gonad and the anchor cell, in the very early L1 stage animals. When the ablated animals are in their L3 molt, we observed their vulval differentiation using Nomarski optics as described above.

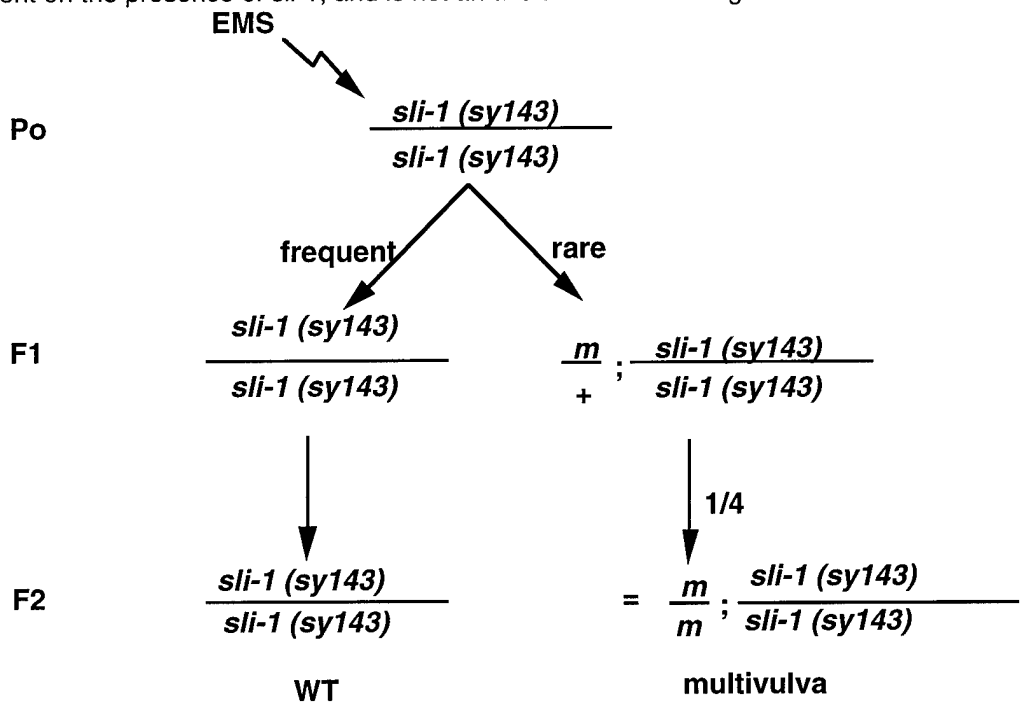
After transferring freshly laid eggs from a culture plate to a new plate, we counted the number of adults after three days. The viability of a mutant strain is calculated as follows. viability (%) = number of adult animals / number of eggs x 100; lethality (%) = 100 - viability (%). The viability of wild-type strain N2 is theoretically 100%, but due to damage during egg transfer, or animals that escape from the plate, the viability of N2 was calculated as 94 %.

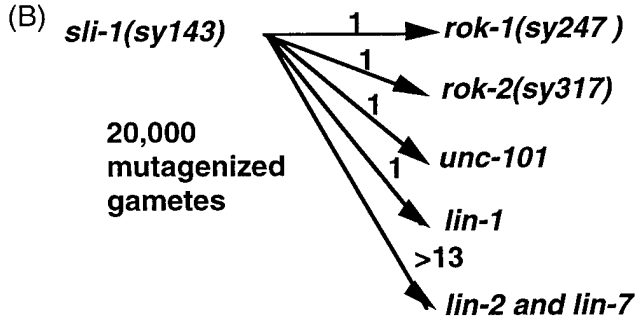
Identification of *rok-1* and *rok-2* as negative regulators of vulval differentiation in *C. elegans*

To identify additional negative regulators of vulval differentiation, we performed a genetic screen in which we mutagenized *sli-1(sy143)* animals, and looked for animals with greater than wild-type vulval differentiation (Figure 2). Since *sli-1(sy143)* suppresses the vulvaless phenotype of *lin-2, lin-7, lin-10,* and *let-23* mutations to a Hin phenotype (Jongeward et al., 1995), and causes a multivulva phenotype with *unc-101* mutations (Yoon et al., 1995), we expected to recover alleles of these genes from our mutagenesis as well as mutations in new genes. Also we expected to recover mutations in any gene downstream in the vulval induction pathway that cause a multivulva phenotype, for example, *lin-1.* Indeed, we recovered eight *lin-2* alleles, five *lin-10* alleles, one *unc-101* allele, one *lin-1* allele as well two mutations that define new genes, *rok-1(sy247)* and *rok-2(sy(317).*

Figure 2. Mutagenesis of *sli-1* homozygotes. (A) Schematic of the mutagenesis procedure. This scheme is for isolating recessive mutations that cause hyperinduction of VPCs in the presence of a *sli-1* mutation. *sli-1(sy143)* animals are mutagenized with EMS as chemical mutagen. Mutated chromosomes exist as heterozygotes in the F1 progeny, and show phenotypes in the F2. EMS = ethyl methane sulfonate, Hin = hyperinduced, WT = wild type, and m= a new mutation. (B). Results of the *sli-1* mutageneses. *lin-2* and *lin-10* mutations were the most frequent mutations recovered. One allele of *lin-1* and *unc-101* were recovered in addition to mutations in two new genes, *rok-*

1 and *rok-2* Specifically, we mutagenized 10,000 F1 chromosome sets of *sli-1(sy143)* by ethyl methane sulfonate (EMS), and screened F2 progeny for mutants with more than wild-type vulval induction (hyperinduced; Hin), which are indicated by the presence of additional bump(s) on the ventral side of the animals which is visible under a dissecting microscope. From the first round of mutagenesis, we analyzed the candidate mutants, and found that *lin-2* and *lin-10* mutations had the highest recovery frequency. Thereafter, we performed the complementation tests with *lin-2* and *lin-10* alleles as soon as we isolated candidate Hin animals to see if they are alleles of these genes. If any candidate complemented *lin-2* and *lin-10* mutations, we proceeded to further map the mutations. We performed backcrosses with either a wild-type (N2) or a *sli-1* strain. Three to four days after crossing N2 or *sli-1(sy143)* males with the Hin animals from the mutagenesis, we transferred many L4 hermaphrodites to a new plate, and the next day we transferred single wild-type animals to new plates. We scored F2 animals for the segregation of wild type, hyperinduced, and vulvaless "bag" phenotypes. If we observed 3/16 of the F2 progeny of N2 backcross were egg laying-defective (Egl) due to a vulvaless phenotype, we concluded that the new mutation is dependent on the presence of *sli-1* mutations and is an allele of a vulvaless gene unlinked to X. If we observed about the same number of F2 Hin animals (1/4) from both N2 and *sli-1* backcrosses and few or no vulvaless animals in N2 backcross, we concluded that either the mutation is dependent on the presence of *sli-1* and linked to X, or the mutation is independent of *sli-1*. If 1/4 of *sli-1* backcross F2 and 1/16 of N2 backcross F2 are Hin, and no F2 animals in N2 backcross are vulvaless, then this mutation is dependent on the presence of *sli-1*, and is not an allele of a vulvaless gene.





Characterization of *rok-1(sy247)*

rok-1(sy247) causes a Hin phenotype in the presence of a *sli-1* mutation. Under Nomarski optics, the average number of VPCs differentiating as vulval in the *rok-1; sli-1* double mutants is 3.8 VPCs, compared with 3.0 of N2 wild type or *sli-1* single mutants. A gonad ablation experiment showed that vulval induction in these Hin animals is still dependent on the presence of the gonad; no vulval induction was observed in four gonad-ablated animals. This gonad-dependency is different from that of *unc-101; sli-1* double mutants, since the *unc-101; sli-1* double mutants still display some vulval induction after gonad ablation (Yoon et al., 1995).

rok-1(sy247) causes additional phenotypes in the presence of a *sli-1* mutation. The double mutants of *rok-1; sli-1* genotype show partial lethality: Some of the progeny of the homozygous mothers died as embryos or early larvae (55.6%, n=261; 15%, n=93; 25% @ 25°C, n=116). We constructed *rok-1(sy247); him-5(e1490); sli-1(sy143)* triple mutants to examine the male phenotype of *rok-1(sy247)* in the presence of *sli-1* mutations. The males display an abnormal spicule structure, and the mating capability is abolished in these males.

We mapped *rok-1(sy247)* using linkage mapping and three factor crosses (Table 2) between *dpy-20* and *unc-31* on the chromosome *IV*. We found that the deficiencies *sDf22* and *sDf60* delete the *rok-1* locus.

Table 2. Genetic mapping of *rok-1(sy247)*: Three factor crosses performed in a *sli-1(sy143)* background. See Materials and Methods for detail. Dpy, dumpy phenotype; nDpy, non-Dpy; Unc, uncoordinated; nUnc, non-uncoordinated. Hin, hyperinduced, indidative of *sy247* intreaction with sli-1.

Genotype of heterozygotes	Phenotype of Recombinants selected	Number of recombinants segregating Hin progeny
dpy-13 unc-24/rok-1	Dpy non-Unc	3/3
unc-22 unc-31	Unc-31 non-Unc-22	0/3
unc-31 dpy-4/rok-1	Unc non-Dpy	0/27
unc-22 dpy-4/rok-1	Dpy non-Unc	4/5
dpy-20 unc-31/rok-1	Dpy non-Unc	12/13
dpy-20 unc-31/rok-1	Unc non-Dpy	1/9

The *rok-1(sy247)* mutation reduces *rok-1* activity. We compared the extent of vulval differentiation in *rok-1(sy247) / rok-1(sy247); sli-1(sy143) / sli-1(sy143)* animals with that in *sDf22 / rok-1(sy247) ; sli-1(sy143) / sli-1(sy143)* animals. We found that the vulval phenotype of *rok-1(sy247) / rok-1(sy247); sli-1(sy143) / sli-1(sy143)* animals is as severe as that of *sDf22 / rok-1(sy247) ; sli-1(sy143) / sli-1(sy143)* animals. We also compared the lethality of the heterozygotes of *sDf22 / rok-1(sy247); sli-1(sy143)* with that of *rok-1(sy247) / rok-1(sy247); sli-1(sy143)* animals. We found that the homozygotes of the genotype *dpy-13 rok-1(sy247) / dpy-13 rok-1(sy247); sli-1(sy143)* has as severe a lethality phenotype as the heterozygotes of the genotype *sDf22 / dpy-13 rok-1(sy247); sli-1* Therefore, the *sy247* mutation reduces the *rok-1* activity.

Experimental details: We used *marker; sli-1* double mutants to map *rok-1(sy247)* by linkage mapping, because *rok-1* needs the presence of *sli-1* mutations to show a Hin phenotype (see Results). For *marker; sli-1* strains, we mated *marker*/+ males with *sli-1(sy143)* hermaphrodites, and obtained *marker* or + / + ; *sli-1*/ ø males. These males were mated with *sli-1* hermaphrodites, and their hermaphrodite progeny were transferred to individual plates and checked for segregation of marker phenotypes. The animals with the marker phenotype are of the genotype *marker; sli-1*. We followed a standard mapping method as described in (Brenner, 1974).

We then used the triple mutants of *dpy-13 unc-24; sli-1, unc-22 dpy-4;sli-1, unc-22 unc-31; sli-1, dpy-20 unc-31; sli-1* and *unc-31 dpy-4; sli-1* strains to map *rok-1(sy247)* by three factor crosses. We mated N2 males with these marker strains, and the male progeny of genotype *marker*/+; *sli-1*/ø were mated into *rok-1(sy247); sli-1(sy143)* hermaphrodites. We isolated many L4 progeny from this mating, isolated recombinant animals in the next generation from the plates that segregated parental markers, and checked the segregation of the Hin phenotype in the next generation. *rok-1* mapped very close to *unc-31*, because from the three factor crosses using *unc-22 unc-31; sli-1* and *unc-31 dpy-4; sli-1*, *rok-1* was inseparable from the *unc-31* locus. None of 3 Unc-31 non-Unc-22 recombinants from *unc-22 unc-31*/ *rok-1* heterozygous mothers, and none of 27 Unc-31 nonDpy-4 recombinants from *unc-31 dpy-4*/ *rok-1* mothers segregated Hin progeny. We then used *dpy-20 unc-31; sli-1* to map further *rok-1(sy247)*. Twelve of thirteen Dpy non-Unc recombinants segregated Hin animals, and 1 out of 9 Unc non-Dpy recombinants segregated Hin progeny. We also performed complementation tests with the deficiencies *sDf22* and *sDf60*.

sDf22 / nT1 heat-shocked males were mated with *dpy-13 unc-24;sli-1* hermaphrodites, and individual male progeny, whose genotype are either *sDf22*/ *dpy-13 unc-24* or *nT1*/ *dpy-13 unc-24*; *sli-1*/ø , from this mating were mated with *dpy-13 rok-1; sli-1* hermaphrodites. Non-Dpy cross-progeny of each mating, whose genotype is either *nT1*/ *dpy-13 rok-1; sli-1* or *sDf22*/ *dpy-13 rok-1; sli-1,* were examined for their vulval induction. From one plate, 9 non-Dpy cross-progeny were obtained, and they showed an average of 3.8 VPCs induced, which is virtually indistinguishable from *rok-1(sy247)* / *rok-1(sy247); sli-1(sy143)*/ *sli-1(sy143)* animals. Moreover, four of nine animals had more than three VPCs induced (44 %), similar to *rok-1(sy247)* / *rok-1(sy247); sli-1(sy143)*/ *sli-1(sy143)* animals. Another plate had three nonDpy progeny with wild-type vulval differentiation, indicating that the genotype of these animals was *nT1*/ *dpy- 13 rok-1; sli-1*. For lethality comparison, we scored the heterozygotes of the genotype *sDf22*/ *dpy-13 rok-1(sy247); sli-1* for their segregation of Dpy and nonDpy progeny. From the heterozygotes of the genotype *sDf22*/ *dpy-13 rok-1(sy247); sli-1*, 271 animals were Dpy, and 139 animals, nonDpy. The ratio of Dpy animals to nonDpy animals is 2:1. If the lethality of heterozygotes of the genotype *sDf22*/ *dpy-13 rok-1(sy247); sli-1* had been enhanced, the ratio of Dpy/nonDpy would have been less than 2.

New *rok-1* alleles. Since *rok-1* in *trans* to a deletion of the locus has a similar phenotype to *rok-1* homozygotes, and the deletion does not enhance the synthetic lethality with *sli-1*, the *sy247* mutation likely severely reduces activity of the *rok-1* locus,

and thus ROK-1 is a negative regulator of LET-23-mediated signaling. Since *rok-1(sy247)/Df; sli-1/sli-1* is viable and has a phenotype, we screened for additional alleles of *rok-1* to facilitate the molecular cloning and to confirm that we are analyzing loss-of-function alleles. We mutagenized *sli-1* males and mated them with *rok-1 unc-31; sli-1* hermaphrodites. We then screened approximately 4000 cross-progeny (non-Unc-31) for multivulva animals. We obtained two such animals, which should be *rok-1(sy247) unc-31/rok-1(new); sli-1/sli-1.* We will retest to confirm that these strains harbor *rok-1* mutations, and map them with respect to *unc-31.* We will sequence the mutant alleles to confirm the molecular cloning.

***rok-1(sy247)* causes a temperature-sensitive hyperinduction of VPCs**

We outcrossed *rok-1(sy247).* At 20°C, we found that *rok-1(sy247)* animals display wild-type vulval differentiation. We obtained *rok-1(sy247)* males by heatshock at 30° C for six hours, and found that they had wild-type spicules and normal mating capability at 20°C. There is no detectable lethality in the single mutant (viability was 91% compared to 94% for N2). Therefore, the *rok-1(sy247)* mutation alone does not cause any visible phenotype at 20°C. At 25°C, however, *rok-1(sy247)* homozygotes show excessive vulval differentiation. Specifically, out of 25 animals observed by Nomarski optics, 5 animals had more than three VPCs induced to become vulval cells. All five animals had P4.p cells induced. The *sy247* allele displays a partially penetrant temperature sensitive multivulva phenotype: at 20°C, none of 22 animals had extra vulval differentiation, while at 25°, an average of 3.1 VPCs induced (n=25). None of eight animals at 20° nor at 25 ° had vulval differentiation in the absence of the gonad.

Table 3. Vulval differentiation of single, double, and triple mutants carrying *unc-101, rok-1,* or *sli-1.* Vulval induction of each animal was examined using Nomarski optics. For intact animals, Average numbers of induced VPCs per animal, and percentage of the animals having more than three induced VPCs are also shown. Wild-type vulval induction is three VPCs per animal. The maximum number of VPCs that can be induced is six. For the gonad-ablated animals, the average numbers of induced VPCs per animal after ablation are shown. The numbers shown in the parentheses are the numbers of animals examined.

	Intact		Gonad-ablated	
Genotype	**Avg. # VPCs Difff**	**% with >3**	**Avg. # VPCs Difff**	
+	3	0	0	(>10)
sli-1	3	0	0	(6)
rok-1	3	0	0	(6)
unc-101	3	0	0	(6)
rok-1; sli-1	3.8	64	0	(4)
unc-101; sli-1	3.8	65	1.9	(7)
unc-101; rok-1	3.5	47	0	(10)
unc-101; rok-1; sli-1	4.9	100	2	(9)

Prior to these experiments, four negative regulatory activities had been identified in the vulval induction pathway: they are represented by *lin-15(A), lin-15(B), sli-1,* and *unc-101.* To examine how *rok-1* interact with other negative regulators, we con-

structed double mutants or triple mutants containing mutations in these negative regulatory genes. The extent of vulval differentiation of these mutants is summarized in Table 3.

unc-101; rok-1 double mutants have an average of 3.5 VPCs induced (n=19). As described above, *rok-1; sli-1* double mutants have an average of 3.8 VPCs induced. *unc-101; sli-1* double mutants show an average of 3.7 VPCs differentiating as vulval (Yoon et al., 1995). Interestingly, *unc-101; rok-1; sli-1* triple mutants show an even greater extent of vulval differentiation (an average of 4.9 VPCs) than any of the three double mutant combinations. When comparing the percentage of Hin animals in these mutants, the enhanced phenotype becomes more obvious. In any single mutants, no animal shows greater than wild-type induction. In double mutants, 47 to 65 % of animals in the populations show greater than wild-type induction, and in the triple mutants, 100 % of animals in the population show greater than wild-type induction. Therefore, *rok-1* defines a new redundant negative regulator of vulval induction, acting together with *unc-101* and *sli-1.*

We examined whether vulval differentiation in the double and triple mutants of *unc-101, sli-1,* and *rok-1* is dependent on the presence of the anchor cell. We ablated the precursor cells of the gonad and the anchor cell, and observed the animals at their L3 molt stages to examine the extent of vulval differentiation (Table 3). In the double mutants of *rok-1; sli-1* and *rok-1; unc-101* , none of the ablated animals displayed vulval differentiation (n=4, and 10, respectively). In the ablated triple mutants of *unc-101; rok-1; sli-1*, the average number of VPCs induced was 2.0 VPCs per animal (n=9), which is virtually identical to that of *unc-101; sli-1* double mutants.

Strain constructions: For *rok-1(sy247)* single mutant strain, we used an *unc-31 dpy-4* chromosome to balance *rok-1(sy247)* , as it maps within 0.2 m.u. of *unc-31.* Single males from the mating between *unc-31 dpy-4/ +* males and *rok-1(sy247) ; sli-1(sy143)* hermaphrodites were mated with *unc-31 dpy-4* hermaphrodites. Males of the genotype *unc-31 dpy-4/ rok-1; sli-1/ø* will generate Unc Dpy male cross progeny from this mating, and males of the genotype *rok-1/ +; sli-1/ø* will not generate any Unc Dpy male cross progeny. From a mating plate with Unc Dpy males, we picked wild-type males whose genotype should be *unc-31 dpy-4/ rok-1; sli-1/ø ,* and mated them with *unc-31 dpy-4* hermaphrodites. We then mated the wild-type male progeny of this mating, whose genotype should be *unc-31 dpy-4/ rok-1; +/ø* , with *unc-31 dpy-4* hermaphrodites. Wild-type cross progeny of from this mating have the genotype of *unc-31 dpy-4/ rok-1; +/+* . Among the progeny of these mothers, we picked many wild-type L4 animals to individual plates. Animals that did not segregate any Unc, Dpy, or Unc Dpy animals were of the genotype *rok-1; +.* We confirmed the presence of *rok-1(sy247)* in this final strain by mating *rok-1(sy247)* hermaphrodites with *sli-1* males and checking segregation of Hin animals in the F2.

For *unc-101; rok-1* double mutants, we isolated non-Unc progeny from the mating of *rok-1(sy247)* males with *unc-101* hermaphrodites, whose genotype is *unc-101/+; rok-1/+.* We observed the Unc progeny of these mothers by Nomarski optics, and found that some of them are Hin. These Hin Unc animals are of the genotype of *unc-101; rok-1.*

For *unc-101; rok-1; sli-1* triple mutants , from the mating of *unc-101*/+ males with *rok-1; sli-1* hermaphrodites, we isolated *unc-101 or +/ +; rok-1/+; sli-1/+* progeny. In the next generation, we selected non-Unc Hin animals from the plate with Unc segregants, and among the progeny of these Hin animals, we isolated Unc animals whose genotype should be *unc-101; rok-1; sli-1.*

For *rok-1; lin-15 (A or B)* double mutants, we used *unc-31 dpy-4; lin-15 (A or B)* strains in the construction. We mated the *rok-1* males with hermaphrodites of the genotype *unc-31 dpy-4; lin-15.* The male progeny from this mating, whose genotype should be *unc-31 dpy-4/ rok-1; lin-15/ø* , were mated again with hermaphrodites of the genotype *unc-31 dpy-4; lin-15.* The non-Unc non-Dpy hermaphrodite cross-progeny from this mating are of the genotype *unc-31 dpy-4 / rok-1; lin-15/lin-15.* Among the progeny of these hermaphrodites, the non-Unc non-Dpy animals that do not segregate any Unc Dpy progeny were of the genotype *rok-1/rok-1; lin-15/lin-15.*

For *let-23(sy1); rok-1(sy247)* double mutants , we first constructed *sy1; unc-31 dpy-4* strains. From the mating of *rok-1(sy247)* males with *sy1; unc-31 dpy-4* hermaphrodites, we isolated cross-progeny whose genotype is *sy1/+; rok-1/unc-31 dpy-4.* We picked many individual non-Unc-31 non-Dpy-4 Vul animals in the next generation, whose genotype could be one of the two classes: *sy1/sy1; rok-1/rok-1* or *sy1/sy1; rok-1/unc-31 dpy-4,* depending on whether *rok-1(sy247)* suppresses the vulvaless phenotype. If *rok-1(sy247)* does not suppress the vulvaless phenotype to wild type or Hin, 1/3 of the non-Unc-31 non-Dpy-4 Vul animals would be of the first class, which is the wanted genotype, and segregate no Dpy Unc animals. The other 2/3 of the non-Unc-31 non-Dpy-4 Vul animals would be of the second class, and segregate Dpy Unc animals. If *rok-1(sy247)* does suppress the vulvaless phenotype, then all the non-Unc-31 non-Dpy-4 Vul animals would be of the second class, and segregate 1/4 of Unc Dpy Vul animals, 2/4 of non-Unc non-Dpy Vul animals that segregate Unc Dpy progeny, and 1/4 of non-Unc non-Dpy non-Vul animals that do not segregate any Unc Dpy animals. Animals of the last category are of the genotype *let-23(sy1); rok-1(sy247).*

For the *unc-101; let-23; rok-1* strain, we mated *let-23(sy1); rok-1(sy247)* males with *unc-101(sy108); rok-1(sy247)* hermaphrodites. From the cross progeny of the mating, whose genotype is *unc-101/+; let-23/+; rok-1/rok-1,* we picked nonUnc vulvaless animals, whose genotype is *unc-101* or +/ +; *let-23/let-23; rok-1/rok-1.* The Unc progeny form these mothers are of the genotype *unc-101; let-23; rok-1.*

For *let-23; rok-1; sli-1* and *unc-101; let-23; rok-1; sli-1* mutants, we mated *let-23(sy1); rok-1(sy247)* males with *unc-101(sy108); rok-1(sy247); sli-1(sy143)*hermaphrodites. From the cross progeny of this mating, whose genotype is *unc-101/+; let-23/+; rok-1/rok-1; sli-1/+,* we picked nonUnc vulvaless animals whose genotype is *unc-101* or + /+; *let-23/let-23; rok-1/rok-1; sli-1* or +/+ (note that some of *let-23; rok-1* double mutants are still vulvaless since the vulvaless phenotype is not completely suppressed by the *rok-1* mutation, see the results for details). Among the progeny of the animals above that segregated Unc progeny, we picked nonUnc hyperinduced animals whose genotype should be *unc-101* or +/ +; *let-23/let-23; rok-1/rok-1; sli-1/sli-1.* The Unc progeny from the above mothers are of the genotype *unc-101; let-23; rok-1; sli-1,* and the animals that do not segregate any Unc progeny are of the genotype *let-23; rok-1; sli-1.*

Lethality. We also compared the lethality associated with variety of single, double, and triple mutants (Table 4). The *sli-1* and *rok-1* mutations do not cause any lethality alone, whereas *unc-101* mutations result in about 45 % lethality: 45 % of the progeny of *unc-101* homozygous mothers die. As described above, 56 % of *rok-1; sli-1* double mutants die before adulthood. Double mutants defective in both *unc-101* and *sli-1* show 54% lethality, which is not significantly different from the *unc-101* single mutants. Therefore, *sli-1* mutations do not enhance the lethality caused by *unc-101* mutations. By contrast, double mutants of *unc-101; rok-1* show enhanced lethality: 82 % lethality (n= 185). Therefore, a *rok-1* mutation enhances the lethality caused by *unc-101* mutations. The lethality of the triple mutants was about 79 % (n= 109), indistinguishable from that of *unc-101; rok-1* double mutants. The lethality of *rok-1; lin-15(A)* or *rok-1; lin-15(B)* was essentially identical to wild type.

Table 4. Viability table of single, double, and triple mutants carrying *unc-101, rok-1, sli-1,* and/or *lin-15A,* and *B.* Viability was calculated as number of adult animals divided by number of eggs picked for examination.

Genotype	Viability %	N
+	94	96
sli-1	92	278
rok-1	100	
unc-101	55	521
rok-1; sli-1	44	261
unc-101; sli-1	ca. 50	
unc-101; rok-1	18	185
unc-101; rok-1; sli-1	21	109

sli-1 was identified by its mutations that suppress the vulvaless phenotype of *let-23* mutations. To test whether *rok-1* can also suppress a mutation in *let-23* gene, an EGFR homolog, we constructed a *let-23(sy1); rok-1(sy247)* double mutant strain. *sy1* is an allele that is suppressed by *unc-101* or *sli-1* mutations from an average of 0.8 VPC induced per animal to 3.5 VPCs per animal. The *let-23(sy1); rok-1(sy247)* double mutants displayed an average of 2.3 VPCs induced per animal. Therefore, *rok-1(sy247)* can suppress the *let-23(sy1)* mutation, though not as well as the other two negative regulator mutations.

As described above, *let-23(sy1)* is the allele that was used for identifying *unc-101* and *sli-1* as negative regulators of vulval differentiation (Jongeward et al., 1995). The *let-23(sy1)* mutant phenotype is suppressed by *unc-101* or *sli-1* mutations from a vulvaless phenotype to not only a wild-type vulva but also greater than wild-type vulval differentiation (Lee et al, 1994; Jongeward et al., 1995). One possible explanation for that is that *let-23(sy1)* is defective both in the positive and negative activity of the LET-23 receptor (Aroian and Sternberg, 1990). To further elucidate the nature of *let-23(sy1)* and its interaction with negative regulators, we examined the phenotypes of a variety of triple and quadruple mutants bearing *let-23(sy1)* and *rok-1, unc-101,* and/or *sli-1* (Table 6). The presence of the *sy1* mutation enhanced the extent of vulval differentiation in all the combination of double and triple mu-

tants bearing *unc-101, sli-1,* and *rok-1* mutations, suggesting that the *sy1* mutation is indeed defective in both the positive and negative role of the receptor. Mutations in negative regulator genes can suppress the reduced positive activity of the LET-23 receptor caused by the *sy1* mutation, and cause synergistic effect with the *sy1* mutation in their negative roles, that is, greater extent of vulval differentiation. It is not clear whether the negative activity stimulated by LET-23 defines another negative regulation pathway. After ablation of the precursors of the gonad and the anchor cell, *unc-101;let-23(sy1); sli-1* and *unc-101; let-23(sy1); rok-1; sli-1* mutant animals displayed partially gonad-independent vulval differentiation.

Table 5. Synergistic interactions of *rok-1, sli-1* and *unc-101.* Vulval differentiation scored in a *let-23(sy1*)background. Average numbers of induced VPCs per animal in the mutant animals bearing *unc-101, rok-1,* and *sli-1* mutations in the *let-23(sy1)* genetic background. The average numbers of induced VPCs per animal with or without the gonad at 20°C are shown.

Genotype (all *let-23(sy1)*)	AC+	AC-
+	0.8	0
unc-101;	3.6	0
rok-1	2.2	0
sli-1	4.3	0
unc-101; lsli-1	4.3	0 (5)
rok-1; sli-1	5.5	0 (8)
unc-101;l sli-1	5.0	1.0
unc-101; rok-1; sli-1	5.5	2.0 (10)

We propose that *rok-1* defines a new negative regulator of the vulval induction pathway. First, the triple mutants of *unc-101; rok-1; sli-1* display a greater extent of vulval differentiation than any double mutant. In these triple mutants, all the animals observed under Nomarski optics showed greater than wild-type differentiation, while in double mutants some animals showed wild-type induction, and some, greater than wild-type differentiation. It is probable that in the triple mutants, the sensitivity of VPCs are greater than that of VPCs in the double mutants where they show fluctuations of induction level in individual animals. Second, *rok-1(sy247)* mutation suppresses the vulvaless phenotype of *lin-7* and *let-23(sy1),* though not as well as the other two negative regulators. Third, the *rok-1(sy247)* mutation alone can cause excessive vulval differentiation at higher temperature (25°C).

The ablation experiments of *unc-101; sli-1, unc-101; rok-1* and *unc-101; rok-1; sli-1* mutants suggest that *rok-1* activity may be involved either in negative regulation of the activity of the vulval induction pathway only after activation by the signal, or in negative regulation of the signal in the anchor cell. We rule out the possibility that *rok-1* is active exclusively in the anchor cell, because the *rok-1; lin-15(A)* double mutant animals still display vulval differentiation after the ablation of the gonad. The fact that both *sli-1* and *rok-1* behaves as if they were class B synmuv genes and that mutations in both the genes still cause an excessive vulval differentiation (note that

mutations in the same class of synmuv genes normally do not cause an excessive vulval differentiation) again suggest that *rok-1* and *sli-1* are in a distinct pathway from *lin-15* and that there may be a quantitative threshold for their activity to be effective for negative regulation.

The fact that any single mutation in these five classes of negative regulator genes does not cause excessive vulval differentiation alone (except for *rok-1* at 25°C) indicates that the activities of these genes have redundant functions that may act to ensure high fidelity. Multistep carcinogenesis is an analogous process, requireing mmultiple mutations in a variety of proto-oncogenes and tumor suppressor genes. In higher organisms, activation of an EGFR pathway by oncogenic mutations is one of the causes of cancer. Similarly, in *C. elegans,* activation of an EGFR pathway by gain-of-function mutations of *lin-3* (EGF homolog), *let-23* (EGFR homolog), and *let-60* (ras homolog) result in excessive vulval differentiation. Thus, these mutations can be considered 'oncogenic' to some extent. It is possible that the negative regulatory genes in vulval induction may help to define novel tumor suppressor genes.

All three double mutant combinations of *unc-101, rok-1,* and *sli-1* are hyperinduced, and the triple mutants show an even higher hyperinduction, suggesting that the activity of each gene is partially independent of those the others so that the loss of activities is additive. By contrast, the lethality associated with *unc-101* single mutants or *unc-101; rok-1* double mutants is not enhanced by the presence of a *sli-1* mutation. A *sli-1* mutation does enhance the lethality of a *rok-1* mutation, suggesting that activity of *sli-1* for survival may be independent of that of *rok-1,* but may be overlapping with that of *unc-101.* The causes of the lethality associated with *sli-1* and *unc-101* mutations and that with a *rok-1* mutation seem to be independent: if these genes are in the same pathway for survival, the lethality should not be increased by adding a *rok-1* mutation. Therefore, these three genes show different redundant aspects in different gene activities. A possible explanation for the different redundancies is that while these genes are involved in the same biochemical pathway in different cells ortissues, cellular processes in these different cells or tissues may have different sensitivities to a loss of activities of these three genes. Another possibility is that these genes are involved in different pathways in different cells for different effects. We hypothesize two different "two out of three" redundancies for three activities required for prevention of excessive vulval induction and survival of animals: two of the three activities may be sufficient for proper function of these systems. We assume that all three genes may act as different activities for regulation of vulval induction. Then any double mutants would be defective in two of the three activities, causing a greater than wild-type vulval differentiation. For survival of an animal, we assume that *unc-101* may be involved in two of the activities, *rok-1* in the third, and *sli-1* in one of the activities in which *unc-101* is involved. If so, *unc-101* single mutants would show some lethality, while the other two single mutants would not, and *unc-101; rok-1* double mutant animals show about the same lethality as *unc-101; rok-1; sli-1* triple mutant animals. There may be other redundant essential genes interacting with *unc-101, rok-1,* and *sli-1,* because all the triple mutants do not die.

Molecular cloning of *rok-1*..

We identifed *rok-1* in a genetic screen for mutations that result in excessive vulval differentiation in a *sli-1* background. *rok-1(sy247)* is silent as a homozygote, but displays excessive vulval induction in combination with a mutation in *sli-1*. In addition, *rok-1; sli-1* double mutants display partial synthetic lethality 15-75% of doubly mutant animals die before adulthood. Thus, *rok-1* and *sli-1* are partially redundant for some functions. Like *sli-1* mutations, *rok-1* mutations suppress a truncated LET-23 mutation that is defective in vulval induction. Since triple mutants defective in *unc-101*, *rok-1* and *sli-1* are more severely affected than any double mutant, we infer that *rok-1* defines another pathway. We therefore sought to molecularly clone *rok-1*.

We mapped *rok-1* to nematode chromosome IV, close and to the left of *unc-31*, which correlate the genetic and physical maps and constrain *rok-1* to a region of approximately 100 kilobases (Fig. 3). This region is covered by 20 cosmid clones with two regions docvered by YAC but not cosmid clones [Coulson et al.,, 1986, 1988, pers. comm.]. We tested six cosmids for their ability to rescue *rok-1* phenotypes (e.g., conferring a multivulva phenotype in combination with a mutation of *sli-1*) in transgenic animals. We found that cosmid C01C7 but not W03F4, F26E1, K09D4, W07C5 or F45E5 rescued a *rok-1* mutation (Table 6). We generated a restriction map of C01C7, and tested subclones for their ability to rescue a *rok-1* mutant. By this method we have identified a 14 kilobase subclone comprising 11.5 kb of nematode genomic DNA that contains *rok-1* (Fig. 4).

Figure 3. Crude physical map of the *rok-1* region of chromsome IV.

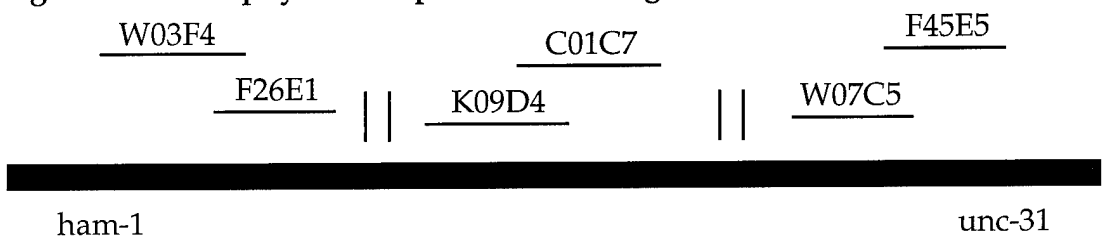
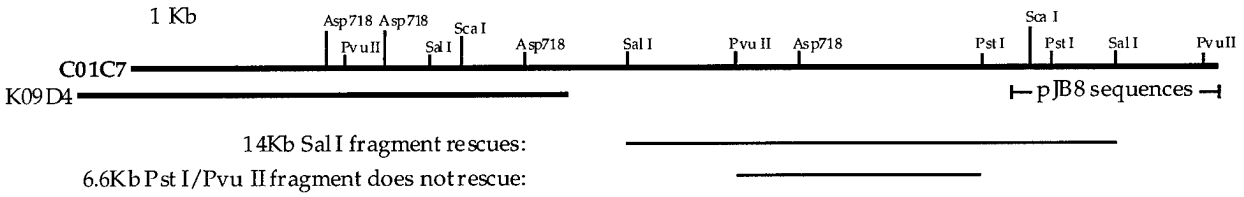


Table 6. Data for rescue of *rok-1* mutant phenotype in transgenic animals. Injection method of Mello et al. (1991). Note that not every trangenic line expresses input DNAs; thus some of the C01C7+K09D4 pooled injections resulted in lines that failed to resuce the *rok-1* mutation. **Injection protocol:** *rok-1(sy247)* is a temperature sensitive mutation: at 20°C 18/38 (47%) *dpy-20, rok-1(sy247); sli-1* animals are multivulva, while at 25°C 30/34 (88%) *dpy-20, rok-1(sy247); sli-1* animals are multivulva. *dpy-20(e1282), rok-1(sy247); sli-1(sy143)* animals were injected with 7.5 ng/µl test cosmid, 15ng/µl pMH86 (rescues *dpy-20;* Han and Sternberg, 1990) made up to a final concentration of 150 ng/µl with pBluescript and incubated at 25°C. Control data: *rok-1(sy247); sli-1(sy143)* animals at 25°C are 11/14 (79%) multivulva and *sli-1(sy143)* animals at 25°C are 0/16 (0%) multivulva.

cosmid injected	Transgenic line	% multivulva	no. animals scored
none	no rescue	79%	
rok-1(+) control	rescue	0%	
W03F4 + F26E1	#1/1	89%	9
(no rescue)	#1/2	67%	6
	#1/3	80%	15
	#1/4	67%	6
	#1/5	82%	28
C01C7 + K09D4	#4/1	0%	22
	#4/2	0%	18
(rescue)	#4/3	0%	14
	#4/4	0%	7
	#4/6	100%	7
	#4/7	0%	24
	#4/8	20%	10
	#4/10	0%	9
	#8/1	10%	10
	#8/2	0%	5
	#8/3	0%	10
	#8/4	10%	10
	#8/5	0%	11
W07F4 + F45E5	#9/1	100%	1
	#9/4	75%	8
(no rescue)	#9/5	75%	12
	#9/6	40%	5
	#9/8	79%	14
C01C7	#3/1	0%	24
(rescue)	#3/2	15%	26
	#3/3	0%	22
K09D4	#1/6	83%	6
	#1/7	78%	9
(no rescue)	#1/9	86%	7
	#1/13	75%	8
	#1/14	100%	6

Figure 4. Restriction map of C01C7 and location of minimal rescuing subclone.



4. Identification and cloning of additional negative regulators

We have carried out one screen for additional genes that interact with *sli-1* (see Fig.2). We discovered a mutation *sy317* that defines a locus we tentatively call *rok-2*. This mutation appears to map to chromosome III, but additional genetic mapping is required to confirm this linkage.

5. Gene interactions

To help elucidate the roles of *sli-1*, *sli-2* and *rok-1* in regulating signaling, we will are using pathway analysis *in vivo*. *sli-1*, *rok-1* and *unc-101* are in distinct pathways because double mutants display more severe phenotypes than the single mutant. To test whether *sli-1* and *sli-2* act in the same pathway, we constructed and examined double mutant strains. The double mutant [*sli-2; sli-1*] has the same phenotype as either single mutants, and we thus conclude that *sli-1* and *sli-2* are in a common pathway. Similarly, we demonstrated that a *rok-1; rok-2* double mutant is more severe than either single mutant. Additional double mutants will be construted with *rok-2*.

lin-15 A* and *lin-15B. The *lin-15* locus encodes two functions, *lin-15A* and *lin-15B*. Certain mutations of *lin-15* abolish functions of both the class A and class B, resulting in a multivulva phenotype. Other mutations in *lin-15*, however, abolish either class A or class B function, resulting in no vulval differentiation defect (Ferguson and Horvitz, 1989). To display a multivulva phenotype, both class A and class B functions must be mutated. No double mutants of the same classes cause vulval defects. *sli-1* can act as a class B multivulva gene at 25°C, as double mutants of genotype *lin-15A; sli-1(sy143)* display a gonad-independent Muv phenotype at 25°C. To examine whether *rok-1* can interact with a *lin-15A* and/or *lin-15B* gene, we constructed a double mutant strain of *rok-1(sy247); lin-15(sy197* or *n744)* . *lin-15(sy197)* is a class A gene and *lin-15(n744)* is a class B gene. The average number of induced VPCs of the *rok-1; lin-15(B)* double mutant animals was 3 at 20°C, and 3.2 (n=21) at 25°C (Table 7). This number is not significantly different from the induction level of *rok-1(sy247)* single mutant. The average number of induced VPCs of the *rok-1; lin-15(A)* was 3.5 at 20°C, and 5.5 at 25°C. Therefore, like *sli-1, rok-1* behaves as if it were a class B gene. The vulval induction of *rok-1; lin-15(n744)* was dependent on the presence of the gonad, because no induction was observed after the ablation of the gonad in these animals. However, the induction of *rok-1; lin-15(sy197)* was partially independent of the presence of the gonad, because an average of 2.4 to 3.2 VPCs were induced even in the absence of the gonad. We note that

if *sli-1* were a typical class B gene, then we would have recovered multiple class A mutations, which we did not.

Table 7. Interaction of *rok-1(sy247)* with *lin-15A* and *lin-15B*. The average numbers of induced VPCs per animal with or without the gonad at 20 or 25°C are shown.

	20°C		25°C	
	AC(+)	AC(-)	AC(+)	AC(-)
rok-1(sy247)	3	0 (8)	3.1	0 (8)
lin-15(A)	3	0 (6)	3	0 (9)
lin-15(B)	3	ND	3	ND
rok-1(sy247); lin-15(A)	3.5	2.4 (5)	5.5	3.2 (16)
rok-1(sy247); lin-15(B)	3	0 (5)	3.2	0 (5)

We propose that *lin-15A, lin-15B, unc-101, sli-1,* and *rok-1* define five negative regulatory activities. A combination of activities of all these negative regulators will ensure that only three VPCs out of six VPCs, which all have the potentials to generate vulval cells, are induced by the LIN-3 signal. One way to differentiate the roles of these negative regulators is to examine their effect on the basal and ligand-stimulated activity of *LET-23* receptor signaling, which could be performed by comparing the extent of vulval differentiation of the animals defective in different sets of genes. *lin-15A* and *lin-15B* regulate the basal activity of the signaling since mutations that delete *lin-15A* and *lin-15B* cause all six VPCs to generate vulval tissue even in the absence of the gonad (Ferguson et al., 1987; Huang et al., 1994; Clark et al., 1994). The combination of the *unc-101* and *sli-1* gene activity may negatively regulate the basal activity of the vulval induction pathway as well as the activated activity of the pathway, while *rok-1* does not seem to be involved in negative regulation of the basal activity. The ablation experiment showed that the double mutants of *unc-101; sli-1* and the triple mutants of *unc-101; rok-1; sli-1* displayed some extent of vulval differentiation (~2 VPCs per animal) after ablation of the entire gonad, whereas the double mutants of *unc-101; rok-1* and *rok-1; sli-1* did not display any vulval differentiation after ablation. We cannot rule out the possibility that *unc-101* and *sli-1* are involved solely in negative regulation of stimulated activity of the LET-23 signaling, because our ablation experiments could not eliminate the expression of *lin-3* in other cells than the anchor cell, for example, the tail cells. *lin-3* may be involved in the specification of the P12 neuroblast fate earlier in development (L. Jiang & P. Sternberg, unpublished obs.). Interestingly, in all the cases where partially gonad-independent vulval differentiation occurs after ablation of the gonad of the mutants bearing *unc-101, sli-1,* and/or *rok-1* mutations, the VPCs located posteriorly are preferentially induced (not shown). We speculate that the VPCs might be so sensitive to the signal that they might be able to respond to the signal from the tail. The *rok-1(sy247); lin-15(sy197)* mutant animals did not show any posteriorly biased induction after gonad ablation, suggesting that *lin-15* is only involved in negative regulation of the basal activity of the receptor signaling, not affecting the sensitivity of VPCs, and that lowering negative regulation of both basal and stimulated activity of the receptor signaling can cause an excessive vulval differentiation. In the ablated animals showing biased induction, they show pair-wise induction bias within P5.p /P6.p and P7.p/P8.p cell pairs: P6.p and P8.p cells are more frequently induced

than P5.p or P7.p cells after gonad ablation. This may implicate different potential sensitivity of anterior and posterior cells in these pairs, as is seen in other nematode species such a *Mesorhabditis* n. sp. (Sommer and Sternberg, 1994).

Table 8. Summary of nteractions among negative regulatory mutants. WT, Double mutant is wild-type; Muv, double mutant is at least partially multivulva; nd, not yet determined.

	lin-15B	***unc-101***	***sli-1***	***sli-2***	***rok-1***	***rok-2***
lin-15A	Muv	WT	Muv [25°]	nd	Muv	nd
lin-15B		WT	WT	nd	WT	nd
unc-101			Muv	nd	Muv	nd
sli-1				WT	Muv	Muv
sli-2					.	.
rok-1						

Overexpression of SLI-1, SLI-2 or ROK-1. We have constructed a *sli-1* cDNA under the control of a heatshock (hsp16) promoter/enhancer (A. Fire, pers. comm.' Stringham and Candido, 1993), and are now constructing transgenic animals.

Progress by task as per original Statement of Work:

A brief description of progress on each task for the first year is listed.

Task 1A. Determine whether SLI-1 truncation decreases or increases activity of the protein as assayed in transgenic animals [months 1-6]. a. construct truncated forms by site-directed mutagenesis. •Two mutant forms of *sli-1* that mimic the structure of oncogenic cbl mutants have been constructed. One is a truncation equivalent to original oncogenic form of cbl, v-cbl; the second is a 17 amino acid deletion corresponding to the mouse cell line form of cbl. **b. establish transgenic lines.** •Transgenic animals carrying these constructs have been made. **c. examine phenotype.** •The 17 amino acid deleted form causes no dominant phenotypes but restores *sli-1(+)* function. [on schedule]

Task 1B. Determine expression of SLI-1 in *C. elegans*
•A *lacZ* and green fluorescent protein (GFP) version of *sli-1* have been constructed and transgenic lines are being established. [on schedule]

Task 2. Molecular cloning of SLI-2 from *C. elegans*. a. Correlation of genetic and physical mapping will identify a region of genomic DNA including the *sli-2* locus and less than 150 kilobases of DNA. [months 1-6]. •*sli-2* has been mapped to a region of 150 kb [on schedule] **b. Microinjection of cloned genomic DNA will be used to define the smallest region of DNA that provides *sli-2* function in transgenic animals [months 7-12].** [behind schedule]

Task 3. Molecular cloning of ROK-1 from *C. elegans*. a. Correlation of genetic and physical mapping will identify a region of genomic DNA including the *rok-1* locus and less than 150 kilobases of DNA. [months 1-3]. •*rok-1* locus mapped between *ham-1* and *unc-31*, a region of 100 kilobases. **b. Microinjection of cloned genomic DNA will be used to define the smallest region of DNA that provides *rok-1* function in transgenic animals [months 4-9].** •Rescue with a cosmid has been successful. A subclone that rescues identfied [on schedule]

Task 4. Identification by genetic screens of new loci.

a. Screen for new mutations, carry out screens in parallel. [months 1-24]. •A modified screen for *sli-1* synthetics has been devised, to lower the background of *lin-2*, *lin-7* and *lin-10* mutants. [on schedule]

b. Genetic mapping and complementation of new mutations, parallel experiments [months 3-27] •A new locus has been identified by its synergism with *sli-1*. This locus, tentatively called "*rok-2(sy317)*" maps to III [on schedule]

Task 5. Examination interactions of genes in vivo [1 month, part-time/experiment] •[on schedule]

Task 7. Introduction of c-cbl cDNA into transgenic nematodes. a. **Construct *sli-1/c-cbl* hybrid genes [months 6-7]. b. Examine phentypes of transgenic animals [months 8-14].** •*sli-1* minigene construction is in progress. [behind schedule]

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